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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

Detection and Treatment of Fibrotic Disorders

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☒ Applicant claims small entity status. See 37 CFR 1.27.

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Respectfully submitted,

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Provisional Patent Application
Docket No. UF-418P

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No. : UF-418P

Applicants : Nasser Chegini, Xiaoping Luo, Li Ding, R. Stan Williams

For : Detection and Treatment of Fibrotic Disorders

MS PROVISIONAL PATENT APPLICATION
Commissioner for Patents
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CERTIFICATE OF MAILING BY EXPRESS MAIL (37 C.F.R. § 1.10)

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I hereby certify that the items listed on the attached Provisional Application and Cover Sheet therefore, with copies as required for authorization for use of Deposit Account No. 19-0065, are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and are addressed to: Mail Stop PROVISIONAL PATENT APPLICATION, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313.

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DESCRIPTION

DETECTION AND TREATMENT OF FIBROTIC DISORDERS

The subject invention was made with government support under a research project supported by the National Institutes of Health Grant No. HD37432.

Background of Invention

Leiomyomas are benign uterine smooth muscle tumors, accounting for more than 30% of hysterectomies performed in the United States annually. Leiomyomas consist mainly of smooth muscle cells of myometrial origin and a network of connective tissue (Anderson, *Semin. Reprod. Endocrinol.,* 1996, 14:269-282; Chegini, *Cytokines and Reproduction,* 1999, 133-162).

Abnormal vagina bleeding, pelvic pain and pelvic masses are among the major symptoms associated with leiomyomas. Leiomyomas are considered to originate from cellular transformation of myometrial smooth muscle cells and/or connective tissue fibroblasts during the reproductive years. The identity of factors that initiate such cellular transformation is not known; however, ovarian steroids are essential for leiomyoma growth, and GnRH anolog (GnRHa) therapy, creating a hypoestrogenic condition, is often used for their medical management (Kettel *et al., Fertil. Steril.,* 1993, 60:642-646; Takeuchi *et al., J. Obstet. Gynecol. Res.,* 2000, 26:325-331). GnRHa-induced leiomyoma regression is accompanied by alterations in uterine arteriole size, blood flow, and cellular content as well as changes in the expression of several growth factors, cytokines, extracellular matrix, proteases, and protease inhibitors (reviewed in Chegini, *Cytokines in Human Reproduction,* 2000, 133-162; Nowak, *Bailliere Best Pract Res. Clin Obstet. Gynaecol.,* 1999, 13:223-238). Differential expression and autocrine/paracrine action of many of these molecules are considered to play a central role in leiomyoma growth and GnRHa-induced regression (Chegini, *Cytokines in Human Reproduction,* 2000, 133-162; Nowak, *Bailliere Best Pract Res. Clin Obstet. Gynaecol.,* 1999, 13:223-238).

At the cellular level, a combination of mitotic activity, cellular hypertrophy, and accumulation of extracellular matrix (ECM) are considered to participate in leiomyoma growth (Anderson, *Semin. Reprod. Endocrinol.,* 1996, 14:269-282; Chegini, *Cytokines and Reproduction,* 1999, 133-162; Stewart *et al., J. Clin. Endocrinal Metab.,* 1994, 79:900-906; Wolanska *et al., Mol Cell Biochem.,* 1998, 189:145-152). Compared to myometrium, leiomyomas are reported to overexpress estrogen and progesterone receptors, and GnRHa therapy lowers their content in both tissues (Stewart *et al., Semin, Reprod. Endocrinol.,* 1995, 10:344-357; Englund *et al., J. Clin. Endocrinol Metab.,* 1998, 83:4092-4092). Clinical and basic science research shows that GnRHa acting through suppression of the pituitary–gonadal axis cause leiomyoma to regress by affecting uterine arteriole size, blood flow at the tumor level. But its effect at cellular and molecular levels in leiomyoma has not been investigated.

Transforming growth factor-beta (TGF-β) is a key regulator of cell growth, differentiation and the expression of extracellular matrix (ECM), adhesion molecules, proteases, and protease inhibitors, which are critical to ECM turnover (Lawrence, *Eur. Cytokine Network,* 1996, 7:363-374; Branton *et al., Microbes Infect.,* 1999, 1:1349-1365). Overproduction of TGFβ is widely accepted as a key element in tissue fibrosis, acting through a mechanism involving enhanced cell migration, expression, and deposition of various ECM with concurrent inhibition of proteases that accelerate ECM degradation (Branton *et al., Microbes Infect.,* 1999, 1:1349-1365). Leiomyoma is a fibrotic disorder in which TGFβ and TGFβ receptors are overexpressed compared with normal unaffected myometrium, by down-regulation of their expression (Dou *et al., J. Clin. Endocrinol. Metab.,* 1996, 81:3222-3230). Under *in vitro* conditions, TGFβ regulates its own expression, the expression of ECM, matrix metalloproteinases, and tissue inhibitor of matrix metalloproteinases as well as the growth of leiomyoma and myometrial smooth muscle cells (Chegini *et al., J. Clin. Endocrinol. Metab.,* 1999, 84:4138-3143; Lee *et al., J. Clin. Endocrinol. Metab.,* 2001, 86:913-920; Arici *et al., Fertil. Steril.,* 2000, 73:1006-1011; Chegini *et al., Mol. Hum. Reprod.,* 2002, 8:1071-1078). In addition, GnRHa treatment has been shown to down-regulate TGFβ- and ovarian steroid-induced TGFβ expression in these cells (Chegini *et al., Mol. Hum. Reprod.,* 2002, 8:1071-1078).

It has been demonstrated that leiomyoma, myometrium and their isolated smooth muscle cells (LSMC and MSMC) express TGF-β and TGF-β receptor mRNA and protein, with overexpression of TGF-β1, TGF-β3, and TGF-β type II receptor in leiomyomas and a marked down regulation in tumors from women who received GnRHa therapy. It has also been discovered that TGF-β is synthesized, retained, and released by LSMC differently than by MSMC. The data show that LSMC express and release significantly more TGF-β1 mRNA and protein than MSMC, whereas retain more TGF-β1. These findings suggest the existence of a regulatory mechanism that may be critical to local availability and autocrine/paracrine action of TGF-β at the tumor level.

Since the gene expression profiles of leiomyoma and myometrium are still lacking, the present inventors have used a DNA microarray to investigate the differential gene expression in treated and untreated leiomyoma and myometrium. Microarrays have been shown to be of great value in understanding the molecular biology of many diseases, and they have been successfully used to classify various tumors based on their clinical phenotype or genetic background. In this experiment, the present inventors have used gene expression profiling to define the biological relationship between TGF-β and GnRH in tumor growth and regression, and try to unveil the complexity of leiomyoma genesis and development.

Brief Summary of Invention

The present invention provides a method for detecting a fibrotic disorder in a subject by: (a) providing a biological sample obtained from the subject (such as endometrium, peritoneal fluid, and/or smooth muscle cells); (b) analyzing the expression of at least one gene that is differentially expressed in the fibrotic disorder of interest as compared to normal tissue (such as myometrium); and (c) correlating the expression of the gene(s) with the presence or absence of the fibrotic disorder in the subject. Examples of reproductive tract disorders include, but are not limited to, leiomyoma, endometriosis, ovarian hyperstimulation syndrome, adhesions, and other tissue fibroses. Fibrosis involves the deposition of large amounts of extracellular matrix molecules, notably collagen. Fibrosis is involved in normal physiological responses (*e.g.*, wound healing) as well as pathophysiological conditions such as renal failure, liver cirrhosis and heart

disease. The compositions and methods of the present invention are useful for detecting or treating abnormal fibrotic changes in the tissue of a subject.

Differentially expressed genes include those which are differentially expressed in a given fibrotic disorder, including but not limited to, docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17

transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; and titin.

In one embodiment, the differentially expressed gene is at least one of CDKN1B, CDKN1C, and CTGF.

The step of analyzing expression of the differentially expressed gene can be performed by quantifying the amount of differentially expressed gene product present in the sample, *e.g.*, by contacting the sample with an antibody that specifically binds the gene product. This step can also be performed by quantifying the amount of a nucleic acid that encodes the gene product present in the sample, *e.g.*, by contacting the sample with a polynucleotide that hybridizes under stringent conditions to the nucleic acid that encodes the gene product. The latter can also be performed using a polymerase chain reaction (PCR), for example.

Preferably, expression of a plurality of differentially expressed genes is analyzed. In this case, step (c) of correlating the expression of the differentially expressed gene with the presence or absence of the fibrotic disorder in the subject can include determining the ratio of two or more differentially expressed gene products in the sample.

In another aspect, the invention features a method for modulating gene expression in fibrotic tissue. This method includes contacting the tissue with an agent that modulates

expression of a differentially expressed gene in the tissue. The fibrotic tissue can be from a subject with leiomyoma, endometriosis, ovarian hyperstimulation syndrome, adhesions, or other tissue fibroses, for example. The agent can be one that specifically binds the product that is expressed by a differentially expressed gene. The agent can also be a nucleic acid that modulates (*i.e.*, increases or decreases) expression of one or more differentially expressed genes in a cell. The agent can also be one that modulates transcription or translation of a nucleic acid encoding the product of one or more differentially expressed genes. Thus, the agent can take the form of a polynucleotide, such as an antisense oligonucleotide. In other variations of this method, the agent can be an ovarian steroid, such as estradiol and medroxyprogesterone actetate. However, the agent is preferably not a hormone, but is nonetheless capable of modulating the expression of one or more genes that is differentially expressed in a fibrotic disorder, such as those genes differentially expressed upon GnRHa therapy.

Brief Description of Drawings

Figure 1 shows hierarchical clustering analysis of the gene expression patterns in untreated Leiomyoma and matched myometrium (UF and UM, N=6), treated leiomyoma and matched myometrium (TF and TM, N=6) ($P<0.02$). The dendrogram on the right of the figure shows the similarity of the expression patterns of the individual genes and the dendrogram on the left of the figure shows the similarity of the expression patterns between tissues. The index for the heat map corresponding to gene expression intensity is shown at the bottom. Two clusters of genes (N=34) that dramatically decrease and increase in treated and untreated group are listed on the left. The genes of the dendogram on the right of the figure are listed in Figure 1 (in order, from top to bottom of the figure).

Figure 2 shows K-means clustering analysis of gene expression patterns after GnRHa($P<0.005$) (A) and TGF-β ($P<0.001$) (B) treatment in LSMC and MSMC. Cells were cultured in 75 cm flask in DMEM/F12 contain 10% FBS medium. After reach visual confluence, cells were starved in the same medium but contain 2% FBS for 24 hours. After starved, cells were treated with GnRHa (0.1 mM) or TGF-β (T) for 0, 2, 4, 6 hour, or cells pretreated with TGF-β type II receptor antisense (A) or sense (S) oligo for 24 hours, then

stimulated with TGF-β for 2 hours. The index for the heat map corresponding to gene expression intensity is shown in the middle.

Figure 3 shows a comparison of relative Real-time PCR expression levels and microarray hybridization levels of CDKN1B, CDKN1C, and CTGF. To validate the findings obtained by cDNA chip analysis, 10 genes were selected based on (i) expression values, (ii) functional classification and (iii) regulation by GnRH and TGF-β mediated actions. The same RNA were used in the real-time PCR reaction. In these graphs, three genes showed the same pattern in microarray analysis and real-time PCR analysis.

Figure 4 shows a scheme of the sample collection (leiomyoma and myometrium), processing, and analysis, as described in the Materials and Methods section.

Detailed Disclosure

Methods involving conventional molecular biology techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed. Sambrook *et al.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; and Current Protocols in Molecular Biology, ed. Ausubel *et al.*, Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates). Various techniques using polymerase chain reaction (PCR) are described, *e.g.*, in Innis *et al.*, PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. Methods for chemical synthesis of nucleic acids are discussed, for example, in Beaucage and Carruthers, Tetra. Letts. 22:1859-1862, 1981, and Matteucci *et al.*, J. Am. Chem. Soc. 103:3185, 1981. Chemical synthesis of nucleic acids can be performed, for example, on commercial automated oligonucleotide synthesizers. Immunological methods (*e.g.*, preparation of antigen-specific antibodies, immunoprecipitation, and immunoblotting) are described, *e.g.*, in Current Protocols in Immunology, ed. Coligan *et al.*, John Wiley & Sons, New York, 1991; and Methods of Immunological Analysis, ed. Masseyeff *et al.*, John Wiley & Sons, New York, 1992. Conventional methods of gene transfer and gene therapy can also be adapted for use in the present invention. See, *e.g.*, Gene Therapy: Principles and Applications, ed. T. Blackenstein, Springer Verlag, 1999; Gene Therapy Protocols (Methods

in Molecular Medicine), ed. P. D. Robbins, Humana Press, 1997; and Retro-vectors for Human Gene Therapy, ed. C. P. Hodgson, Springer Verlag, 1996.

The following publications are specifically incorporated herein by reference in their entirety, including all figures, tables, and sequences, to the extent they are not inconsistent with the explicit teachings of this specification: U.S. patent publication US 2003/0032044 (Chegini *et al.*), filed July 17, 2002; international publication WO 03/007685 (Chegini *et al.*), filed July 17, 2002; international publication WO 00/20642 (Chegini *et al.*), filed October 1, 1999; U.S. patent publication US 2003/0077589 (Hess-Stumpp *et al.*), filed September 25, 2001; and U.S. patent publication US 2001/0002393 (Palmer *et al.*), filed December 20, 2000.

I. Detecting Fibrotic Disorders

The invention provides a method for detecting a fibrotic disorder in the tissue of a subject. This method includes the steps of: (a) providing a biological sample obtained (*i.e.*, derived) from the subject (such as endometrium or peritoneal fluid); (b) analyzing the expression of a differentially expressed gene in the sample; and (c) correlating the expression of the differentially expressed gene with the presence or absence of the fibrotic disorder in the subject.

Examples of reproductive tract disorders include, but are not limited to, leiomyoma, endometriosis, ovarian hyperstimulation syndrome, adhesions, and other tissue fibroses (*e.g.*, fibroids) (Smits G. *et al.*, N. Engl. J. Med., 2003, 349(8):760-766; Elchalal U. *et al.*, Human Reproduction, 1997, 12(6):1129-1137; Stewart E. *et al.*, Human Reproduction Update, 1996, 2(4):295-306; Shozu M. *et al.*, The Journal of Clinical Endocrinology & Metabolism, 86(11):5405-5411; Estaban J. *et al.*, Arch. Pathol. Lab. Med., 1999, 123:960-962; Lee W. *et al.*, The Korean Journal of Pathology, 2003, 37:71-73; and Kurioka H. *et al.*, Human Reproduction, 1998, 13(5):1357-1360).

Differentially expressed genes include those which are differentially expressed in a given fibrotic disorder, including but not limited to, docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17

(sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25

(mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; and titin.

In one embodiment, the differentially expressed gene is at least one of CDKN1B, CDKN1C, and CTGF.

Suitable subjects for use in the invention can be any human or non-human animal. For example, the subject can be a female animal, such as mammal, like a dog, cat, horse, cow, pig, sheep, goat, chicken, primate, rat, or mouse. Because the experiments presented herein relate to human subjects, a preferred subject for the methods of the invention is a human, such as a human female. Particularly preferred are female subjects suspected of having or at risk for developing a fibrotic disorder within the reproductive tract, *e.g.*, a woman suspected of having or at risk for developing leiomyoma, endometriosis, or peritoneal adhesions based on clinical findings or other diagnostic test results.

The step of providing a biological sample obtained from the subject can be performed by conventional medical techniques. For example, an endometrial tissue sample can be taken from the subject by biopsy. As another example, a sample of peritoneal fluid can be taken from a subject by conventional techniques. Suitable methods are described in more detail in the Examples sections presented below.

The step of analyzing the expression of a differentially expressed gene in the sample can be performed in a variety of different ways. Numerous suitable techniques are known for analyzing gene expression. For example, gene expression can be determined directly by assessing protein expression of cells or fluid of a biological sample (*e.g.*, endometrial tissue or peritoneal fluid). Proteins can be detected using immunological techniques, *e.g.*, using antibodies that specifically bind the protein in assays such as immunofluorescence or immunohistochemical staining and analysis, enzyme-linked immunosorbent assay (ELISA),

radioimmunoassay (RIA), immunoblotting (*e.g.*, Western blotting), and like techniques. Expression of differentially expressed genes can also be determined by directly or indirectly measuring the amount of mRNA encoding protein in a cellular sample using known techniques such as Northern blotting and PCR-based methods such as competitive quantitative reverse transcriptase PCR (Q-RT-PCR). Suitable methods for analyzing expression of differentially expressed genes are described below; nonetheless, other suitable methods might also be employed.

The step of correlating the expression of the gene with the presence or absence of the fibrotic disorder in the subject involves comparing the level of gene expression in the test biological sample with levels of gene expression in control samples, *e.g.*, those derived from subjects known to have or not to have the particular disorder. Thus, after quantifying gene expression in a biological sample from a test subject, the test result is compared to levels of gene expression determined from (a) a panel of cells or tissues derived from subjects (preferably matched to the test subject by age, species, strain or ethnicity, and/or other medically relevant criteria) known to have a particular disorder and (b) a panel of cells or tissues derived from subjects (preferably also matched as above) known not to have a particular disorder. If the test result is closer to the levels (*e.g.*, mean or arithmetic average) from the panel of cells or tissues derived from subjects known to have a particular disorder, then the test result correlates with the test subject having the particular disorder. On the other hand, if the test result is closer to the levels (*e.g.*, mean or arithmetic average) from the panel of cells or tissues derived from subjects known not to have a particular disorder, then the test result correlates with the test subject not having the particular disorder.

II. Modulating Gene Expression

The present invention also provides a method for modulating the expression of genes that are differentially expressed in fibrotic tissues, compared to normal tissues. Restoration of gene expression to levels associated with normal tissue is expected to ameliorate at least some of the symptoms of the fibrotic disorder. This method includes the step of contacting the tissue with an agent that modulates expression of one or more differentially expressed genes in the tissue.

Differentially expressed genes include those which are differentially expressed in a given fibrotic disorder, including but not limited to, docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin,

gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; and titin.

In one embodiment, the differentially expressed gene is at least one of CDKN1B, CDKN1C, and CTGF.

The tissue for use in this method can be any derived from a human or non-human animal. In some embodiments, the tissue is derived from a female reproductive system, *e.g.*, endometrium, or tissue derived from the uterus, cervix, vagina, fallopian tube, or ovary. Because the experiments presented herein relate to human subjects, a preferred tissue sample for the methods of the invention is one derived from a human. Particularly preferred is tissue derived from a subject suspected of having or at risk for developing a fibrotic disorder (such as a woman suspected of having or at risk for developing leoimyoma, endometriosis, ovarian hyperstimulation syndrome, peritoneal adhesions, or other tissue fibroses) based on clinical findings or other diagnostic test results.

The method of the present invention utilizes one or more agents that modulate expression one or more differentially expressed genes in the tissue. Numerous agents for modulating expression of such genes in a tissue are known. Any of those suitable for the particular system being used may be employed. Typical agents for modulating expression of such genes are proteins, nucleic acids, and small organic or inorganic molecules such as hormones (*e.g.*, natural or synthetic steroids). Preferably, the agent is not a hormone.

An example of a protein that can modulate gene expression is an antibody that specifically binds to the gene product. Such an antibody can be used to interfere with the interaction of the gene product and other molecules that bind the gene product. Products of the differentially expressed genes (or immunogenic fragments or analogs thereof) can be used to raise antibodies useful in the invention. Such gene products (*e.g.*, proteins) can be produced by purification from cells/tissues, recombinant techniques or chemical synthesis as described above. Antibodies for use in the invention include polyclonal antibodies, monoclonal antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, and molecules produced using a Fab expression library. See, for example, Kohler *et al.*, Nature 256:495, 1975; Kohler *et al.*, Eur. J. Immunol. 6:511, 1976; Kohler *et al.*, Eur. J. Immunol. 6:292, 1976; Hammerling *et al.*, "Monoclonal Antibodies and T Cell Hybridomas," Elsevier, N.Y., 1981; Ausubel *et al.*, supra; U.S. Patent Nos. 4,376,110, 4,704,692, and 4, 946,778; Kosbor *et al.*, Immunology Today 4:72, 1983; Cole *et al.*, Proc. Natl. Acad. Sci. USA 80:2026, 1983; Cole *et al.*, "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc., pp. 77-96, 1983; and Huse *et al.*, Science 246:1275, 1989.

Other proteins that can modulate gene expression include variants of the gene products that can compete with the native gene products for binding ligands such as naturally occurring receptors of these gene products. Such variants can be generated through various techniques known in the art. For example, protein variants can be made by mutagenesis, such as by introducing discrete point mutation(s), or by truncation. Mutation can give rise to a protein variant having substantially the same, or merely a subset of the functional activity of a native protein. Alternatively, antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to another molecule that interacts with the protein. In addition, agonistic (or superagonistic) forms of the protein may be generated that constitutively express one or more functional activities of the protein. Other variants of the gene products that can be generated include those that are resistant to proteolytic cleavage, as for example, due to mutations which alter protease target sequences. Whether a change in the amino acid sequence of a peptide results in a protein variant having one or more functional activities of a native protein can be

readily determined by testing the variant for a native protein functional activity (*e.g.*, binding a receptor or inducing a cellular response).

Another agent that can modulate gene expression is a non-peptide mimetic or chemically modified form of the gene product that disrupts binding of the encoded protein to other proteins or molecules with which the native protein interacts. See, *e.g.*, Freidinger *et al.* in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (*e.g.*, see Huffman *et al.* in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey *et al.* in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopepitides (Ewenson *et al.* (1986) J. Med. Chem. 29:295; and Ewenson *et al.* in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, Ill, 1985), beta-turn dipeptide cores (Nagai *et al.* (1985) Tetrahedron Lett 26:647; and Sato *et al.* (1986) J. Chem. Soc. Perkin. Trans. 1:1231), and beta-aminoalcohols (Gordon *et al.* (1985) Biochem. Biophys. Res. Commun. 126:419; and Dann *et al.* (1986) Biochem. Biophys. Res. Commun. 134:71). Proteins may also be chemically modified to create derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of proteins encoded by differentially expressed genes can be prepared by linking the chemical moieties to functional groups on amino acid side chains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

The agent that directly reduces expression of the differentially expressed gene can also be a nucleic acid that reduces expression of the gene. For example, the nucleic acid can be an antisense nucleic acid that hybridizes to mRNA encoding the protein. Antisense nucleic acid molecules for use within the invention are those that specifically hybridize (*e.g.* bind) under cellular conditions to cellular mRNA and/or genomic DNA encoding a protein in a manner that inhibits expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix.

Antisense constructs can be delivered as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes the protein. Alternatively, the antisense construct can take the form of an oligonucleotide probe generated *ex vivo* which, when introduced into a protein expressing cell, causes inhibition of protein expression by hybridizing with an mRNA and/or genomic sequences coding for the protein. Such oligonucleotide probes are preferably modified oligonucleotides that are resistant to endogenous nucleases, *e.g.* exonucleases and/or endonucleases, and are therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see, *e.g.*, U.S. Pat. Nos. 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol *et al.*, Biotechniques 6:958-976, 1988; and Stein *et al.*, Cancer Res. 48:2659-2668, 1988. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, *e.g.*, between the -10 and +10 regions of a protein encoding nucleotide sequence, are preferred.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to mRNA encoding the protein to be inhibited. The antisense oligonucleotides will bind to mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, *e.g.*, the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well (Wagner, R., Nature 372:333, 1994). Therefore, oligonucleotides complementary to either

the 5 or 3 untranslated, non-coding regions of a differentially expressed gene could be used in an antisense approach to inhibit translation of endogenous mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5', 3' or coding region of the mRNA, antisense nucleic acids should be at least eighteen nucleotides in length, and are preferably less than about 100 and more preferably less than about 30, 25, 20, or 18 nucleotides in length.

Antisense oligonucleotides of the invention may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxyethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouricil, beta-D-galactosylqueosin- e, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-idimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopenten- yladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Antisense oligonucleotides of the invention may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose; and may additionally include at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet a further embodiment, the antisense oligonucleotide is an alpha-anomeric oligonucleotide. An alpha-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual beta-units, the strands run parallel to

each other (Gautier *et al.*, Nucl. Acids Res. 15:6625-6641, 1987). Such oligonucleotide can be a 2'-0-methylribonucleotide (Inoue *et al.*, Nucl. Acids Res. 15:6131-6148, 1987), or a chimeric RNA-DNA analogue (Inoue *et al.*, FEBS Lett. 215:327-330, 1987).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.* by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein *et al.* (1988) Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451, 1988).

The antisense molecules should be delivered into cells that express the differentially expressed (*e.g.*, overexpressed) genes *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA into cells. For instance, antisense molecules can be introduced directly into the tissue site by such standard techniques as electroporation, liposome-mediated transfection, CaCl-mediated transfection, or the use of a gene gun. Alternatively, modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be used.

However, because it is often difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs, a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong promoter (*e.g.*, the CMV promoter). The use of such a construct to transform cells will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous gene transcripts and thereby prevent translation of the mRNA.

Ribozyme molecules designed to catalytically cleave target mRNA transcripts can also be used to prevent translation of mRNA and expression of protein (see, *e.g.*, PCT Publication No. WO 90/11364, published Oct. 4, 1990; Sarver *et al.*, Science 247:1222-1225, 1990 and U.S. Pat. No. 5,093,246). While ribozymes that cleave mRNA at site-specific recognition sequences can be used to destroy target mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead

ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, Nature 334:585-591, 1988. Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the mRNA; *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts. Ribozymes within the invention can be delivered to a cell using a vector.

The expression of endogenous genes that are overexpressed in fibrotic disorders can also be reduced by inactivating or "knocking out" the gene or its promoter using targeted homologous recombination. See, *e.g.*, Kempin *et al.*, Nature 389: 802 (1997); Smithies *et al.*, Nature 317:230-234, 1985; Thomas and Capecchi, Cell 51:503-512, 1987; and Thompson *et al.*, Cell 5:313-321, 1989. For example, a mutant, non-functional gene variant (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous gene (either the coding regions or regulatory regions of the gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the gene *in vivo*.

Alternatively, endogenous gene expression may be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene(s) (*i.e.*, the gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene in target cells. (See generally, Helene, C., Anticancer Drug Des. 6(6):569-84, 1991; Helene, C., *et al.*, Ann. N.Y. Acad. Sci. 660:27-36, 1992; and Maher, L. J., Bioassays 14(12):807-15, 1992).

Antisense nucleic acid, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramide chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase

promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Another agent that can be used to modulate gene expression in fibrotic tissue is a hormone. Numerous naturally occurring and synthetic hormones are known to cause physiological changes in such tissue and are available commercially. See, *e.g.*, PDR: Physician's Desk Reference, 2002. Those particular hormones which modulate expression of differentially expressed genes in a given sample tissue can be determined empirically by contacting a series of tissue samples with a panel of different hormones and analyzing the tissue samples for changes in phenotype over time. In experiments relating to the invention, it was shown that GnRHa therapy modulated the expression of 297 genes in leiomyoma and myometrium compared to untreated group ($P<0.02$). In addition, GnRHa, TGF-b and TGF-b receptor type II antisense treatments resulted in differential regulation of 134, 144, and 154 specific genes, respectively ($P<0.005$ and 0.001). The products of these genes were functionally categorized as key regulators of cell cycle, transcription factors, signal transduction, ECM turnover and apoptosis. Based on (i) expression values, (ii) functional classification and (iii) regulation by GnRH and TGF-b mediated actions, we selected 10 of these genes and validated their expression in leiomyoma and myometrium, and in LSMC and MSMC using RealTime PCR, western blotting and immunohistochemistry. In conclusion, the results provide additional evidence for the difference in gene expression profile between leiomyoma and myometrium, and reveal the profile of previously unrecognized novel genes whose expression are the target of GnRH and TGF-β actions in leiomyoma and myometrium.

The agent that can be used to modulate gene expression in fibrotic tissue may be administered to non-human animals or humans in pharmaceutically acceptable carriers (*e.g.*, physiological saline) that are selected on the basis of mode and route of administration and standard pharmaceutical practice. For example, the pharmaceutical compositions of the invention might include suitable buffering agents such as acetic acid or its salt (1-2% w/v); citric acid or its salt (1-3% w/v); boric acid or its salt (0.5-2.5% w/v); succinic acid; or phosphoric acid or its salt (0.8-2% w/v); and suitable preservatives such as benzalkonium chloride (0.003-0.03%

w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) or thimerosal (0.004-0.02% w/v). Examples of compositions suitable for parenteral administration include sterile aqueous preparations such as water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils might be used as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Carrier formulations suitable for local, subcutaneous, intramuscular, intraperitoneal or intravenous administrations may be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. The pharmaceutical compositions useful in the invention may be delivered in mixtures of more than one pharmaceutical composition.

The compositions of the invention may be administered to animals or humans by any conventional technique. Such administration might be parenteral (*e.g.*, intravenous, subcutaneous, intramuscular, or intraperitoneal introduction). Preferably, the compositions may also be administered directly to the target site (*e.g.*, a portion of the reproductive tract or peritoneal cavity) by, for example, surgical delivery to an internal or external target site, or by catheter to a site accessible by a blood vessel. Other methods of delivery, *e.g.*, liposomal delivery or diffusion from a device impregnated with the composition, are known in the art. The composition may be administered in a single bolus, multiple injections, or by continuous infusion (*e.g.*, intravenously or by peritoneal dialysis).

The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of response without causing clinically unacceptable adverse effects. Preferred modes of administration include parenteral, injection, infusion, deposition, implantation, anal or vaginal supposition, oral ingestion, inhalation, and topical administration. Injections can be intravenous, intradermal, subcutaneous, intramuscular, or interperitoneal. For example, the pharmaceutical composition can be injected directly into target site for the prevention of fibrotic disorders, such as leiomyoma, endometriosis, ovarian hyperstimulation syndrome, or adhesion formation. In some embodiments, the injections can be given at multiple locations. Implantation includes inserting implantable drug delivery systems, *e.g.*, microspheres, hydrogels, polymeric reservoirs,

cholesterol matrixes, polymeric systems, *e.g.*, matrix erosion and/or diffusion systems and non-polymeric systems, *e.g.*, compressed, fused, or partially fused pellets. Inhalation includes administering the pharmaceutical composition with an aerosol in an inhaler, either alone or attached to a carrier that can be absorbed. For systemic administration, it may be preferred that the pharmaceutical composition is encapsulated in liposomes. The term "parenteral" includes subcutaneous injections, intravenous, intramuscular, intraperitoneal, intrastemal injection or infusion techniques. In certain preferred embodiments of the invention, the administration can be designed so as to result in sequential exposure of the pharmaceutical composition over some period of time, *e.g.*, hours, days, weeks, months or years. This can be accomplished by repeated administrations of the pharmaceutical composition, by one of the methods described above, or alternatively, by a sustained-release delivery system in which the pharmaceutical composition is delivered to the subject for a prolonged period without repeated administrations. By sustained-release delivery system, it is meant that total release of the pharmaceutical composition does not occur immediately upon administration, but rather is delayed for some period of time. Release can occur in bursts or it can occur gradually and continuously. Administration of such a system can be, *e.g.*, by long-lasting oral dosage forms, bolus injections, transdermal patches, and subcutaneous implants.

A therapeutically effective amount is an amount that is capable of producing a medically desirable result in a treated animal or human. As is well known in the medical arts, dosage for any one animal or human depends on many factors, including the subject's size, body surface area, age, the particular composition to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Toxicity and therapeutic efficacy of the compositions of the invention can be determined by standard pharmaceutical procedures, using cells in culture and/or experimental animals to determine the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Agents that exhibit large therapeutic indices are preferred. While agents that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of the tissues to be treated in

order to minimize potential damage to uninvolved tissue and thereby reduce side effects. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within the range of circulating concentrations that include an ED50 with little or no toxicity. The dosage may vary within this range depending on the dosage form employed and the route of administration utilized.

As used herein, the terms "bind," "binds," or "interacts with" mean that one molecule recognizes and adheres to a particular second molecule in a sample, but does not substantially recognize or adhere to other structurally unrelated molecules in the sample. Generally, a first molecule that "specifically binds" a second molecule has a binding affinity greater than about 10^5 to 10^6 moles/liter for that second molecule.

By reference to an "antibody that specifically binds" another molecule is meant an antibody that binds the other molecule, and displays no substantial binding to other naturally occurring proteins other than those sharing the same antigenic determinants as other molecule. The term "antibody" includes polyclonal and monoclonal antibodies as well as antibody fragments or portions of immunolglobulin molecules that can specifically bind the same antigen as the intact antibody molecule.

As used herein, a "nucleic acid," "nucleic acid molecule," "oligonucleotide," or "polynucleotide" means a chain of two or more nucleotides such as RNA (ribonucleic acid) and DNA (deoxyribonucleic acid).

The term "subject," as used herein, means a human or non-human animal, including but not limited to mammals, such as a dog, cat, horse, cow, pig, sheep, goat, chicken, primate, rat, and mouse.

The term "differentially expressed gene", as used herein, means a gene that is either over-expressed or underexpressed in fibrotic tissue, compared to normal, non-fibrotic tissue. Accordingly, the method of treatment of the present invention is directed to upregulating the expression of one or more genes that are underexpressed in fibrotic tissue and downregulating the expression of one or more genes that are overexpressed in fibrotic tissue.

When referring to a differentially expressed gene, the phrase "modulates the expression of" means upregulates or downregulates the amount or functional activity of the gene, or otherwise modifies the availability of the gene product to interact with a receptor.

The terms, "treat", "treatment", and "treating", as used herein, are intended to include the prevention of a fibrotic disorder and partial or full alleviation of an existing fibrotic disorder within a subject.

Materials and Methods

Leiomyoma and matched normal myometrium from patients who received GnRHa therapy and untreated controls (N=6), were collected from premenopausal women who were scheduled to undergo hysterectomy for symptomatic uterine leiomyomas at the University of Florida affiliated Shands Hospital with the approval of the Institutional Review Board. To maintain a standard, leiomyomas used in this study were between 2 to 3 cm in diameter. Following collection, the tissues were processed for preparation of isolated smooth muscle cells. LSMC and MSMC were cultured in DMEM/F12 with 10% FBS medium in 75-cm^2 flasks, when reach visual confluence. Cells were washed and starved in 2% FBS DMEM/F12 for 24 hrs. After that the cells were treated with GnRHa (0.1 mM) or TGF-β (2.5 ng/ml) for 0, 2, 6, 12 hrs, or cell were pretreated with TGF-β Type II Receptor antisense (1 mM) or sense (1 mM) for 24 hrs, following by (2.5 ng/ml) for 2, 6, 12 hrs. All the tissues and the cells were submitted to RNA isolation, as shown in Figure 4.

Double stranded cDNA was generated from 20 mg of total RNA for each of the time points. The cDNA then underwent an *in vitro* transcription reaction to yield biotin-labeled cRNA. The cRNA was fragmented at 94° C for 35 minutes. The fragmented cRNA was then mixed with the hybridization controls (bacterial, eukaryotic) and the mix was hybridized to the gene chip microarray for 16 hours at 45° C. The array was then washed, stained, and scanned. The values obtained from the scanning were used for the analysis of gene expression (see Figure 4).

Example —Differential Gene Expression in Leiomyoma and Normal Myometrium

Gonadotropin releasing hormone analogue (GnRHa) therapy causes leiomyoma regression, accompanied by down-regulation of transforming growth factor beta (TGF-β), a key regulator of tissue fibrosis. The objective of this study was to further investigate the molecular mechanism of how GnRH and TGF-β influence leiomyoma growth and regression. Cohorts of leiomyoma and matched normal myometrium from patients who received GnRHa therapy and untreated controls (N=6), as well as smooth muscle cells (LSMC and MSMC) isolated from these tissues were subjected to gene microarray analysis. Total RNA isolated from these tissues as well as LSMC and MSMC treated with GnRH (0.1 mM), TGF-β (2.5 ng/ml) and TGF-β antisense/sense (1 mM) for 2, 6 and 12 hrs were subjected to Affymetrix HG-U95Av2 arrays (N=65) representing approximately 12,000 unique genes. The expression values were normalized and subjected to unsupervised hierarchical clustering and principal component analysis and analysis of variance to identify the specific gene expression profiles. Based on the above analysis, 105 differentially expressed and regulated genes were identified in leiomyoma compared to myometrium at a significance level of $P<0.02$. GnRHa therapy resulted in alteration of the expression of 297 genes in leiomyoma and myometrium compared to the untreated group ($P<0.02$). In addition, GnRHa, TGF-β and TGF-β receptor type II antisense treatments resulted in differential regulation of 134, 144, and 154 specific genes, respectively ($P<0.005$ and 0.001). The products of these genes were functionally categorized as key regulators of cell cycle, transcription factors, signal transduction, ECM turnover and apoptosis. Based on (i) expression values, (ii) functional classification and (iii) regulation by GnRH and TGF-β mediated actions, 10 of these genes were selected and their expression was validated in leiomyoma and myometrium, and in LSMC and MSMC using RealTime PCR, western blotting and immunohistochemistry. The results are shown in Figures 1-3. The genes of the right dendrogram of Figure 1 are as follows (from top to bottom of the Figure 1):

docking protein 1, 62 kD (downstream of tyrosine kinase 1)

centromere protein A (17 kD)

catenin (cadherin-associated protein), beta 1 (88 kD)

nuclear receptor subfamily 1, group I, member 2

v-rel avian reticuloendotheliosis viral oncogene homolog A
LGN Protein
CDC28 protein kinase 1
hypothetical protein
solute carrier family 17 (sodium phosphate), member 1
FOS-like antigen-1
nuclear matrix protein p84
LERK-6 (EPLG6)
visinin-like 1
phosphodiesterase 10A
KH-type splicing regulatory protein (FUSE binding protein 2)
Polyposis locus (DP1 gene) mRNA,
microtubule-associated protein 2
CDC5 (cell division cycle 5, S pombe, homolog)-like
Centromere autoantigen C (CENPC) mRNA
RNA guanylyltransferase and 5'-phosphatase
Nijmegen breakage syndrome 1 (nibrin)
ribonuclease, RNase A family, 4
keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)
basic helix-loop-helix domain containing, class B, 2
dual specificity phosphatase 1
annexin A11
putative receptor protein
Human endogenous retrovirus HERV-K(HML6)
mitogen-activated protein kinase kinase kinase 12
TXK tyrosine kinase
kynureninase (L-kynurenine hydrolase)
ubiquitin specific protease 4 (proto-oncogene)
peroxisome biogenesis factor 13

olfactory receptor, family 2, subfamily F, member 1
membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)
origin recognition complex, subunit 1 (yeast homolog)-like
dTDP-D-glucose 4,6-dehydratase
cytochrome c oxidase subunit VIa polypeptide 2
gamma-tubulin complex protein 2
Monocyte chemotactic protein-3
myelin transcription factor 1
inhibitor of growth family, member 1-like
thyroid hormone receptor, alpha myosin-binding protein C, slow-type
fragile X mental retardation 2
sonic hedgehog (Drosophila) homolog
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2
SFRS protein kinase 2
excision repair cross-complementing rodent repair deficiency
cyclin-dependent kinase 5, regulatory subunit 1 (p35)
poly(A)-specific ribonuclease (deadenylation nuclease)
solute carrier family 12 (potassium/chloride transporters), member 4
Pseudogene for metallothionein
natriuretic peptide precursor A

intercellular adhesion molecule 2
apoptosis antagonizing transcription factor
similar to rat HREV107
major histocompatibility complex, class II, DP beta 1
MpV17 transgene, murine homolog, glomerulosclerosis
uroporphyrinogen decarboxylase
proteasome (prosome, macropain) 26S subunit, ATPase, 1
fms-related tyrosine kinase 3 ligand

actin, gamma 1
Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
pyruvate kinase, muscle
telomeric repeat binding factor 2
cell division cycle 2, G1 to S and G2 to M
ADP-ribosylation factor 3
NRF1 Protein
H factor (complement)-like 3
serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6
mRNA of muscle specific gene M9
solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3
ribosomal protein L36a
suppressor of Ty (S. cerevisiae) 4 homolog 1
amino-terminal enhancer of split
ubiquitin A-52 residue ribosomal protein fusion product 1
hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase
chaperonin containing TCP1, subunit 2 (beta)
tyrosine kinase with immunoglobulin and epidermal growth factor homology
domains
Fc fragment of IgG, receptor, transporter, alpha
NRD1 convertase
ADP-ribosylation factor 5
transcription elongation factor A (SII), 1
like mouse brain protein E46
titin

These results provide additional evidence for the difference in gene expression profile between leiomyoma and myometrium, and reveal the profile of previously unrecognized novel

genes whose expression are the target of GnRH and TGF-β actions in leiomyoma and myometrium.

In summary, *in vivo* leiomyoma displays a different gene expression profile from matched myometrium, and treated leiomyoma and myometrium also show a gene expression pattern change compared with untreated leiomyoma and matched myometrium. *In vitro* GnRH and TGF-β cause gene expression profile changes in a time-dependent way. TGF-β type II receptor antisense can partly reverse the effect TGF-β. *In vivo* and *in vitro* genes that change significantly were functionally categorized as key regulators of cell cycle, transcription factors, signal transduction, ECM turnover and apoptosis. These results will facilitate the understanding of leiomyoma genesis and development.

All patents, patent applications, provisional applications, and publications referred to or cited herein, whether supra or infra, are incorporated by reference in their entirety, including all figures, tables, and sequences, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

Abstract

The present invention provides a method for detecting a fibrotic disorder in a subject by: (a) providing a biological sample obtained from the subject (such as endometrium, peritoneal fluid, and/or smooth muscle cells); (b) analyzing the expression of at least one gene that is differentially expressed in the fibrotic disorder of interest; and (c) correlating the expression of the gene(s) with the presence or absence of the fibrotic disorder in the subject. The present invention also provides a method and compositions for modulating the expression of genes that are differentially expressed in fibrotic tissues, compared to normal tissues. Restoration of gene expression to levels associated with normal tissue is expected to ameliorate at least some of the symptoms of the fibrotic disorder. This method includes the step of contacting the tissue with an agent that modulates expression of one or more differentially expressed genes in the tissue.

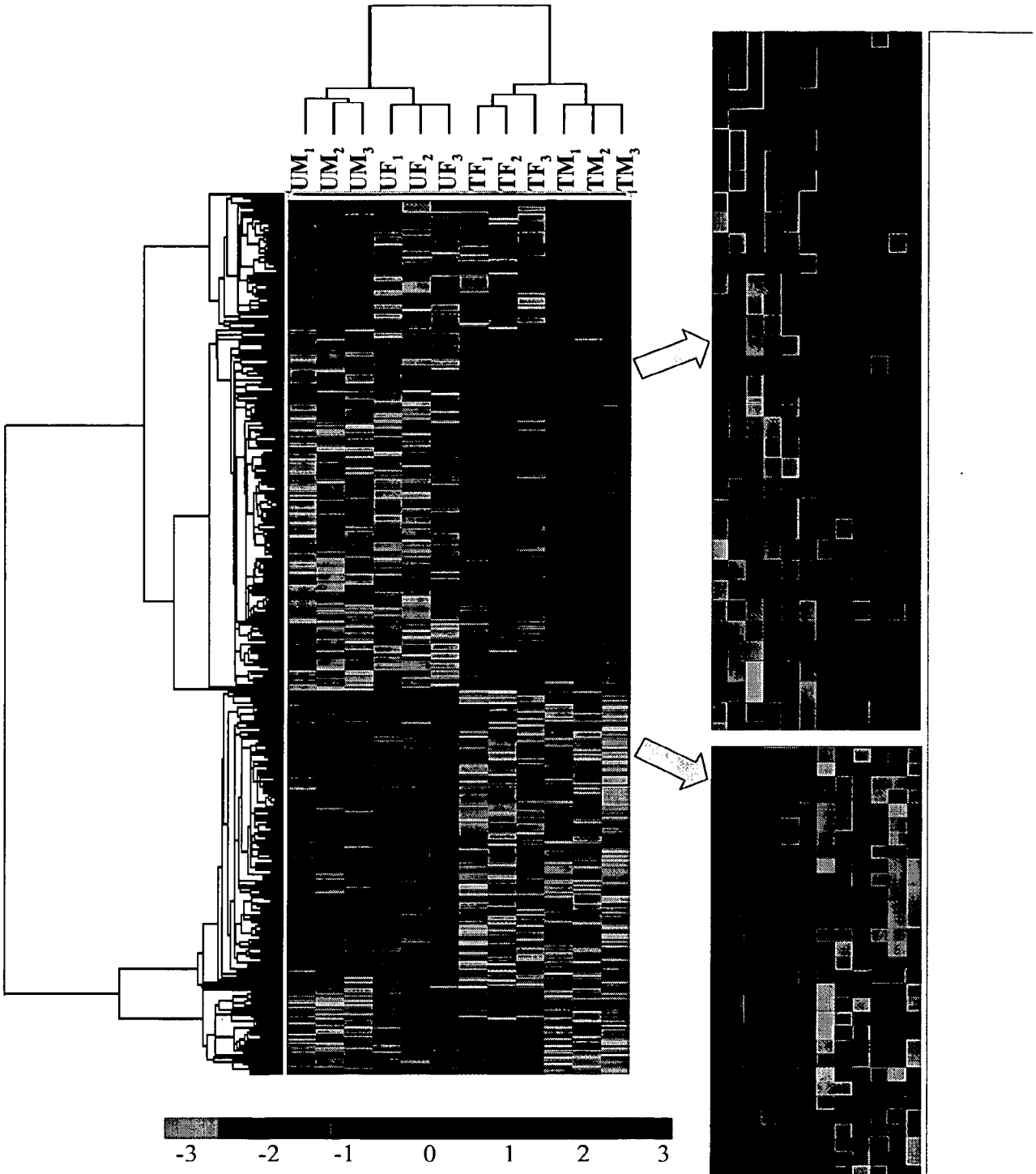

FIG. 1

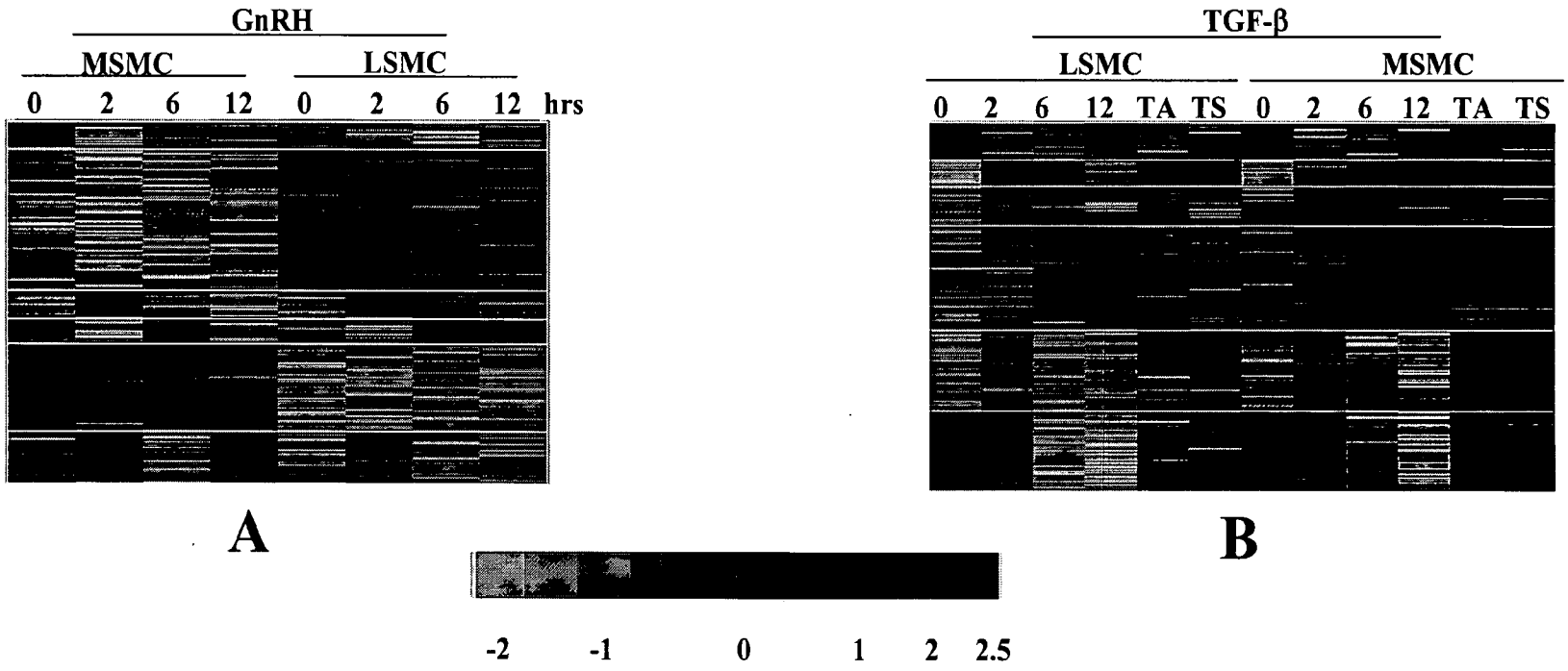
GnRH
MSMC
LSMC
0 2 6 12 0 2 6 12 hrs
A
TGF-β
LSMC
MSMC
0 2 6 12 TA TS 0 2 6 12 TA TS
B
-2 -1 0 1 2 2.5

FIG. 2

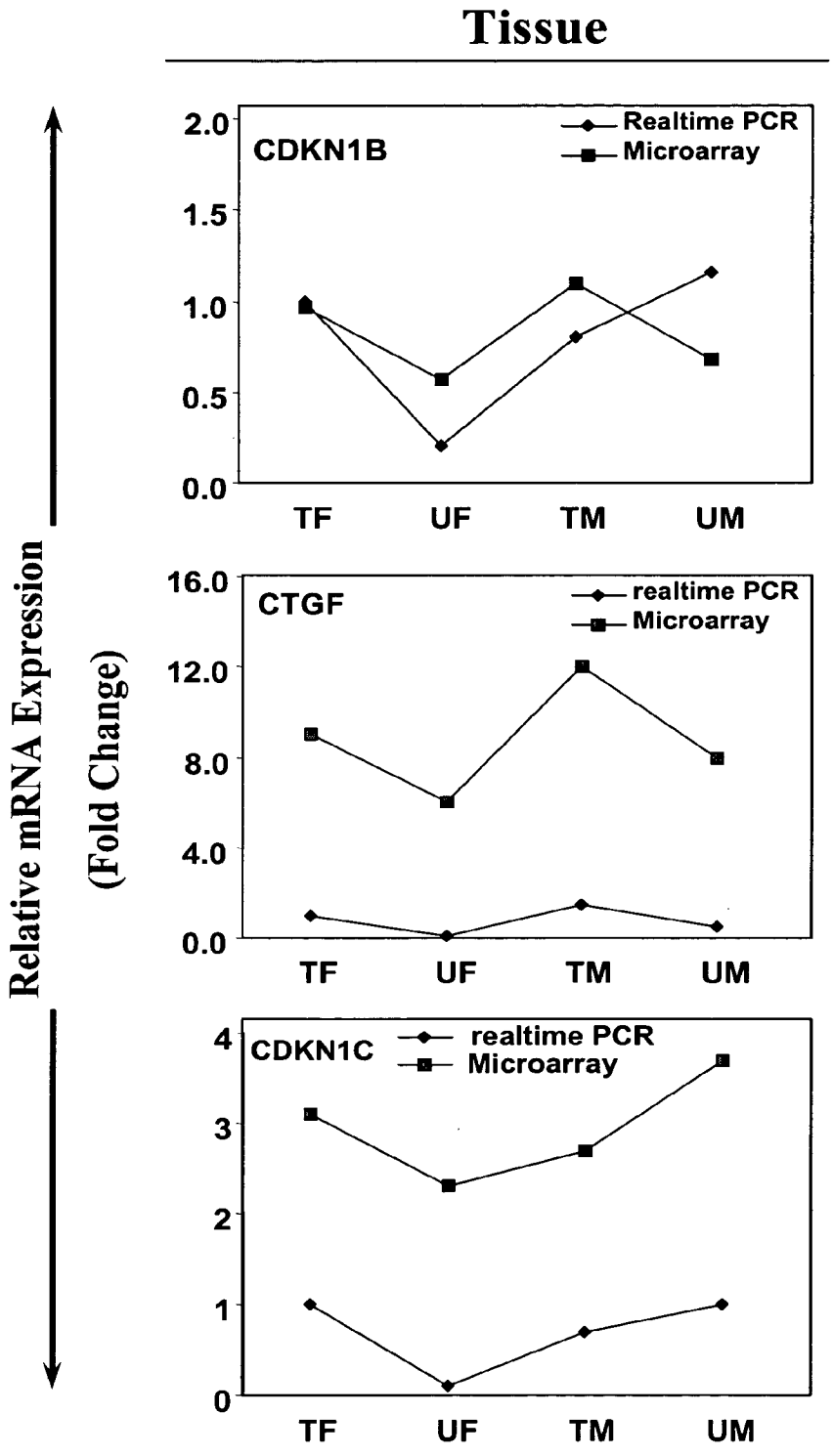
Tissue
Relative mRNA Expression
(Fold Change)
CDKN1B
Realtime PCR
Microarray
2.0
1.5
1.0
0.5
0.0
TF
UF
TM
UM
CTGF
realtime PCR
Microarray
16.0
12.0
8.0
4.0
0.0
TF
UF
TM
UM
CDKN1C
realtime PCR
Microarray
4
3
2
1
0
TF
UF
TM
UM

FIG. 3A

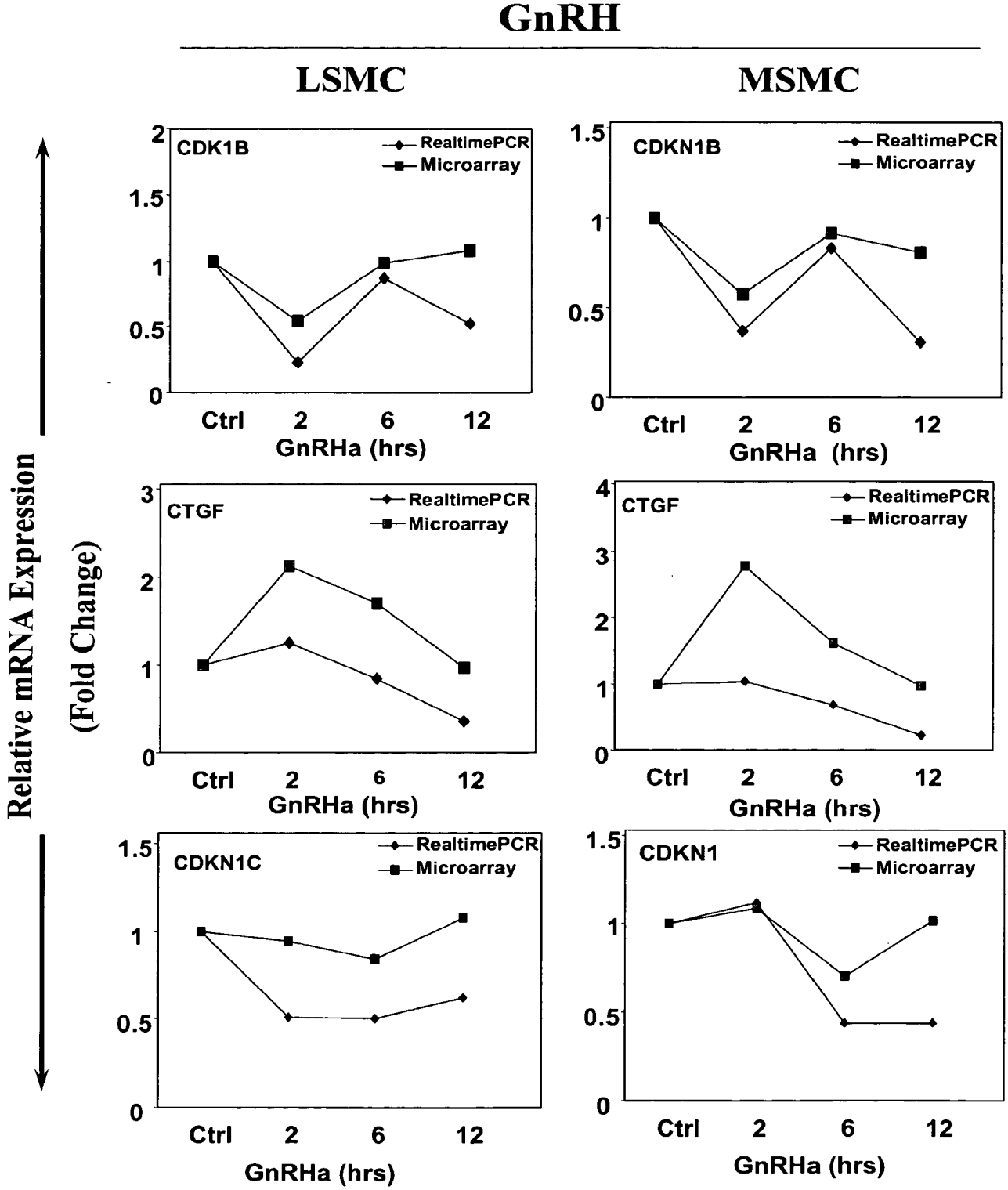
GnRH
LSMC
MSMC
Relative mRNA Expression
(Fold Change)
CDK1B
CDKN1B
CTGF
CTGF
CDKN1C
CDKN1
RealtimePCR
Microarray
Ctrl
2
6
12
GnRHa (hrs)

FIG. 3B

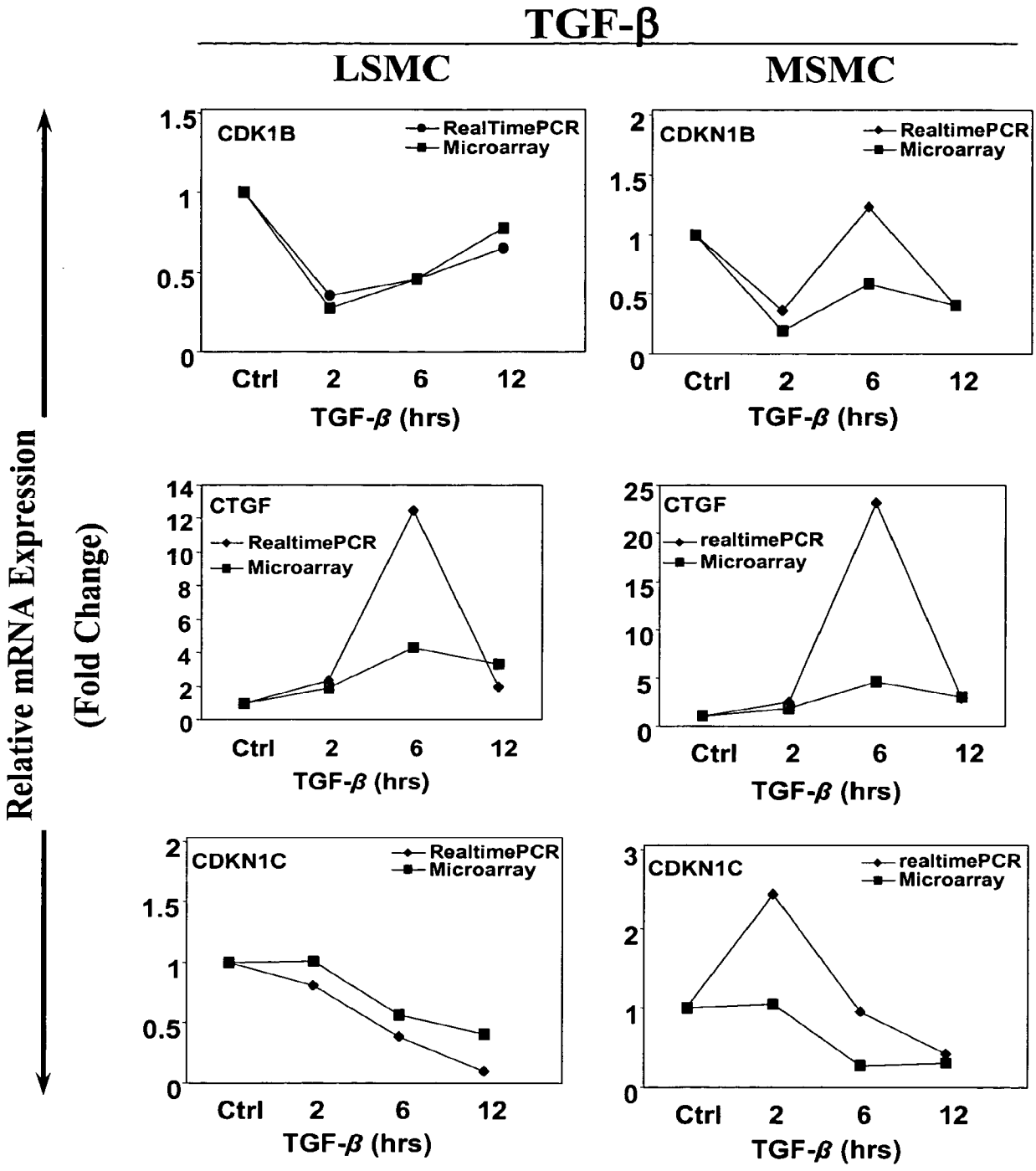
TGF-β
LSMC
MSMC
Relative mRNA Expression
(Fold Change)
CDK1B
RealTimePCR
Microarray
CDKN1B
RealtimePCR
Microarray
CTGF
RealtimePCR
Microarray
CTGF
realtimePCR
Microarray
CDKN1C
RealtimePCR
Microarray
CDKN1C
realtimePCR
Microarray
Ctrl
2
6
12
TGF-β (hrs)

FIG. 3C

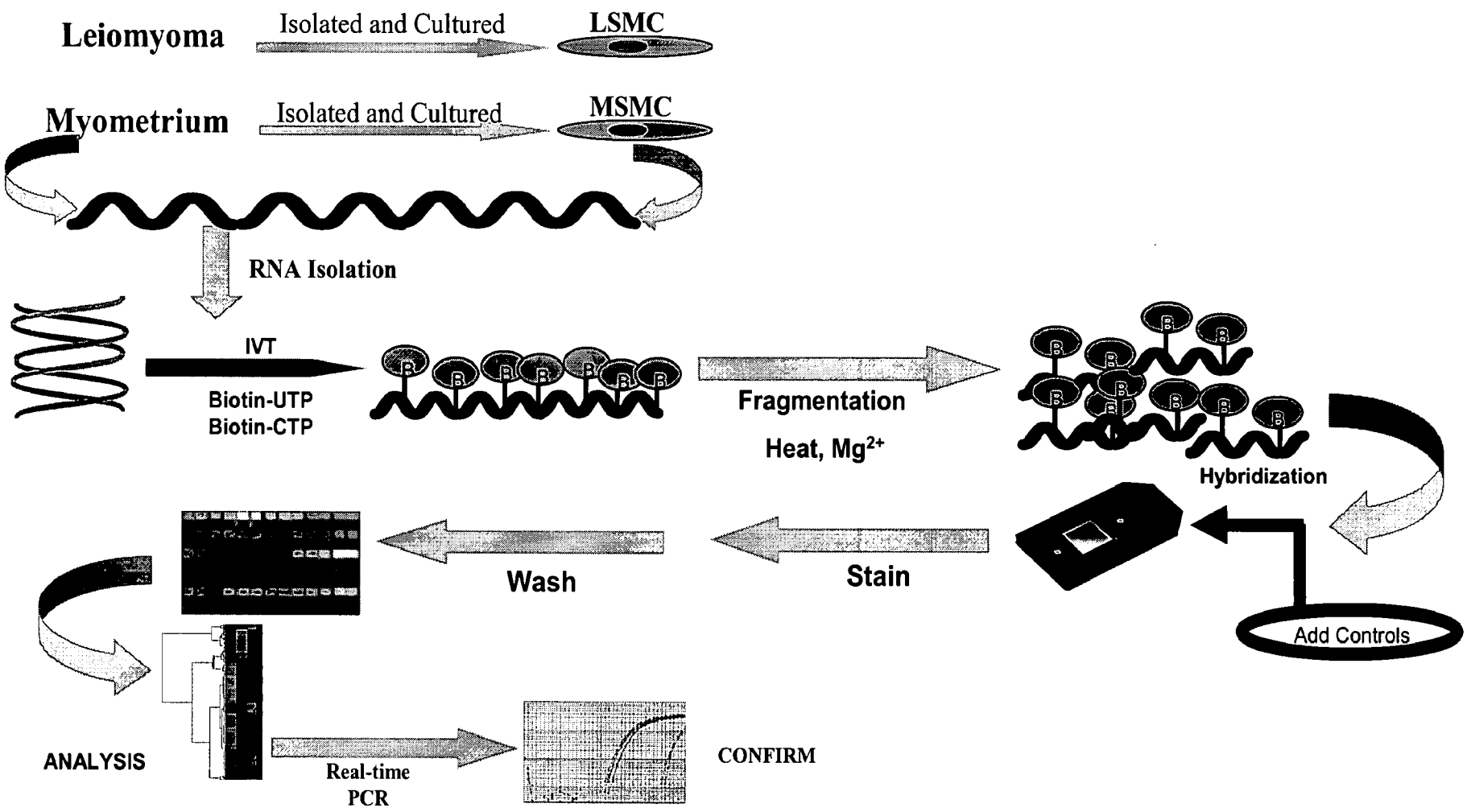
Leiomyoma
Isolated and Cultured
LSMC
Myometrium
Isolated and Cultured
MSMC
RNA Isolation
IVT
Biotin-UTP
Biotin-CTP
Fragmentation
Heat, Mg2+
Hybridization
Add Controls
Stain
Wash
ANALYSIS
Real-time
PCR
CONFIRM

FIG. 4